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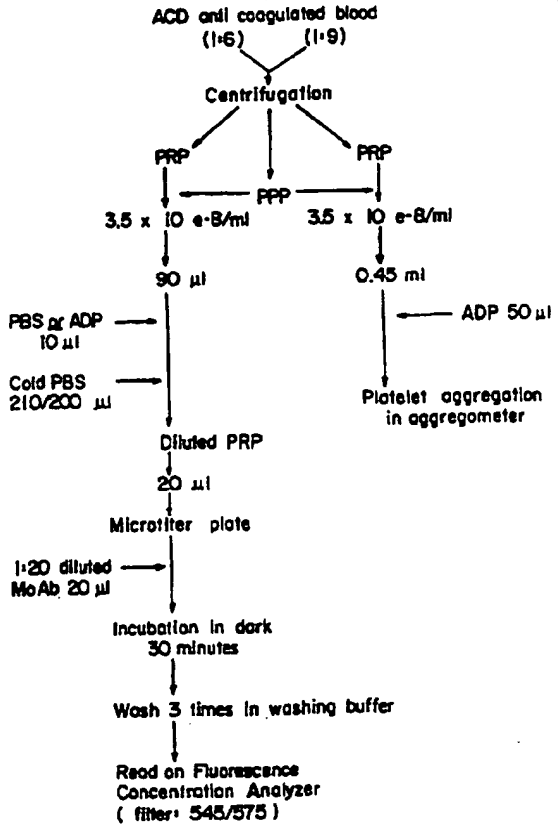
(54) Title: DIRECT FLUORESCENCE-CONJUGATED IMMUNOASSAY FOR PLATELET ACTIVATION

(57) Abstract

A method is provided to measure the extent of platelet activation by fluorometrically determining the extent of expression of P-selectin in a sample of whole blood *in vitro*, using a maximally activated platelet sample as a reference standard.



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DIRECT FLUORESCENCE-CONJUGATED IMMUNOASSAY FOR PLATELET ACTIVATION

Background of the Invention

P-selectin, also known as granule membrane protein-140 (GMP-140), or PADGEM protein, is an integral membrane glycoprotein found in secretory granules of both platelets and endothelial cells. See E.I.B. Peerschke, Am. J. Clin. Pathol., 98, 455 (1992). After activation of these cells by agonists such as thrombin, it is rapidly redistributed to the cell surface during degranulation. P-selectin belongs to the cell surface during degranulation. P-selectin belongs to the selectin family of vascular cell surface receptors that share sequence similarity and overall domain organization. See G.I. Johnston et al., Cell, 56, 1033 (1989). The other known selectins are ELAM-1, a cytokine-inducible endothelial cell receptor for neutrophils, and a leukocyte surface structure which plays a role in directing the homing of lymphocytes to high endothelial venules of peripheral lymph nodes. It has recently been shown by J.-G. Geng et al., Blood, 74, 65a (1989), that human neutrophils bind in a Ca^{2+}-dependent manner to purified P-selectin immobilized on plastic. Furthermore, adhesion of neutrophils to endothelium stimulated with rapid activators such as histamine is mediated at least in part by P-selectin. P-selectin is also involved in binding of activated platelets to monocytes and neutrophils. See, S.A. Hamburger et al., Blood, 75, 550 (1990) and E. Larsen, Cell, 59, 305 (1989).

Because platelet activation accompanies a number of vascular disorders such as unstable angina, peripheral vascular disease, stroke, and procedures such as angioplasty and coronary thrombolysis, considerable effort has been exerted during the last two decades to develop more sensitive and specific methods to detect activated, circulating platelets. See, for example, C.W. Hamm et al., J. Am. Coll. Cardiol., 10, 998 (1987); D.J. Fitzgerald et al., Circulation, 77, 142 (1988) and A.H. Gershlick, Circulation, 81, 128 (1991). The most reliable markers of *in vivo* platelet activation have been substances released from platelets after activation, which can be measured in the plasma or urine: platelet factor 4 (PF4), ß-thromboglobulin (ß-TG), and metabolites of thromboxane A_2. These markers have not achieved widespread clinical acceptance, however, because of technical limitations pertaining to sample collection, processing, and analysis.

Several changes in surface membrane glycoprotein expression can be detected during platelet activation with specific murine monoclonal antibodies. For example, as reported by S.J. Shattil, in Blood, 70, 307 (1987), and C.S. Abrams et al., Blood, 75, 128 (1990), changes in the conversion of the GPIIb-IIIa complex to a functional fibrinogen receptor can be detected. J.N. George et al., J. Clin. Invest., 78, 340 (1986) reported that platelet activation with accompanying alpha granule release can be ascertained by examining P-selectin expression. Thus, assays have been designed that combine the use of activation-specific monoclonal antibodies with flow cytometry. See, for example, R.E. Scharf et al., Arteriosclerosis and Thrombosis, 12, 1475 (1992). These assays can be performed on whole blood and can facilitate the detection of platelet subpopulations that are heterogeneous with respect to their activation status. However, flow-cytometry requires expensive instrumentation, complex data processing and is not practical either to process large numbers of samples economically or to derive results within the timeframe required to affect clinical outcomes in acute situations such as those mentioned above.

Therefore, a need exists for a sensitive, simple and rapid *in vitro* assay to detect the extent of platelet activation.

Summary of the Invention

The present invention provides a method to determine the extent of mammalian platelet activation. In commonly assigned U.S. Patent Application Serial No. 08/142,766, filed October 26, 1993, an assay for platelet activation was disclosed which involved pre-isolation of platelets *in vitro* , i.e., in platelet-rich plasma. Although this method greatly increased the accuracy and rapidity of the assay for activated platelets, the method of the present invention constitutes an improvement over this method in that it may be practiced on whole blood without a pre-isolation step, thus further reducing the processing time. Furthermore, cell loss that may result due to pre-washing and centrifugation is eliminated.

In the method of the present invention, a sample of whole blood is obtained from a patient whose level of platelet activation is to be determined and divided into two portions. One control sample is treated with an activation agonist to maximally activate the platelets contained therein, employing a suitable agonist such as ADP, while the other sample is not treated with exogenous activation agonists. Next, both samples are treated with a prefixing solution, such as paraformaldehyde, and

allowed to incubate for a period of time sufficient to partially fix the platelets. As used herein, the term "partially fixed" indicates a state in which the platelets contained in said samples will not be further activated or damaged by vortexing in the subsequent step, while the red blood cells in the sample will maintain their ability to react with the lytic agent subsequently employed. While undergoing vortexing, an erythrocytic lytic agent is added to the samples. After allowing a sufficient time for the lysis of the erythrocytes to occur, a leukocyte stabilizer is added to stop the lysis reaction. Subsequently, an amount of a cell membrane fixative is added to fully fix and to stabilize the samples. As used herein, the term "stabilize" indicates a state in which the samples can be stored for up to at least about 72 hours before completing the analysis.

Anti-P-selectin antibody is then added to each sample in an amount effective to bind to the activated platelets in each sample. The antibody-activated platelet complexes in each sample are determined fluorometrically, by means of a fluorescent label that is attached to the anti-P-selectin antibody, or by addition to the complexes of a fluorescent label which binds to a binding site on the bound antibody. A ratio of the fluorescence of the complexed activated platelets in the sample not exogenously activated to the fluorescence of the maximally activated platelets provides a measure of the extent of platelet activation in the mammalian donor of the platelets.

Thus, the present invention provides a fluorescence-conjugated immunobinding assay (FCIBA), for measuring endogenous platelet activation comprising:

(a) obtaining a sample of whole blood from a patient whose platelet activation is to be determined and dividing it into a first sample and a second sample;

(b) adding an amount of an activation agonist, such as adenosine 5'-diphosphate (ADP), to said first sample for a period of time effective to maximally activate the activatable platelets in said first sample; while maintaining the second sample for an equivalent period of time;

(c) adding an amount of a solution effective to stop the platelet activation reaction, such as paraformaldehyde, to said first sample for a period of time effective to partially fix the activated platelets in said first sample, while maintaining the second sample for an equivalent period of time;

(d) Vortexing the samples, and sequentially adding:

(i) an amount of an erythrocytic lytic agent effective to lyse the red blood cells in the samples;

(ii) an amount of a leukocyte stabilizer effective to stop the action of the lytic agent; and

(iii) an amount of a cell membrane fixative effective to completely fix stabilize the platelets;

(e) forming binary labelled complexes with the activated platelets in each sample by adding to each sample an amount of

(i) an anti-P-selectin antibody conjugated to a fluorescent label; or

(ii) an anti-P-selectin antibody conjugated to a binding site for a detectable fluorescent label followed by a detectable fluorescent label which specifically binds to said binding site; and

(f) determining the fluorescence of the binary labelled complexes in each sample, wherein a ratio of the fluorescence of said second sample to said first sample provides a measure of the extent of a platelet activation in said second sample.

As used herein, the phrase "endogenous platelet activation" is defined to mean that the activation measured is due to *in vivo* activation and is not due to the addition of exogenous activating agents to the sample *in vitro*. In a preferred embodiment of the invention, step (d) is performed by placing the samples in a Q-Prep machine (Coulter® Corporation, Hialeah, FL) and performing a 30 second cycle. The Q-Prep instrument consists of a matched, three reagent system that provides a gentle, no-wash erythrocyte lysing and fixing system which maintains leukocyte morphology and cell surface integrity. Previous to the development of this assay, the Q-prep machine had been used primarily to prepare white blood cells for flow cytometry. Surprisingly, although the treatment of the platelets in the Q-prep machine fixes them so that the platelets are stable for an extended period of time, the platelets are still able to react with antibodies. The use of whole blood processed in this manner offers an alternative to the use of PRP, since the results obtained using whole blood that has been treated in the Q-prep machine correlate to those obtained using PRP. See Examples 7 and 8.

To develop the assays, platelet samples were activated with various doses of ADP and fixed platelets were incubated with a fluorescence-conjugated anti-P-selectin antibody in wells of microtiter plates. The fluorescence intensity was read on a fluorescence concentration analyzer. Once the platelet samples were fixed, the data collection/analysis procedures can be completed in less than two hours. The intra-assay coefficient of variation (CV) was 6.97%, the time-based inter-assay CV was 6.17%. The present assay demonstrates an excellent correlation ($r = 0.936$) with flow cytometry in the measurement of expressed P-selectin in platelets of 20 normal donors.

Unexpectedly, the translocation of P-selectin in platelets in response to increasing doses of ADP occurred in a dose-dependent manner and correlated positively with ADP-induced platelet aggregation in the aggregometer on the basis of both stimulating doses of ADP ($r = 0.99$) and on the basis of time intervals ($r = 0.92$).

The amount of fibrinogen detected on the surface of the platelets was also increased in response to ADP, whereas the intensity of bound antibodies to the GP IIb-IIIa complex underwent little alteration. In activated platelets, the intensity of fibrinogen antibody binding was correlated with the intensity of P-selectin antibody binding ($r = 0.85$). Thus, in another aspect of the present invention, the measurement of the available sites for fibrinogen binding on platelets may be used to determine the extent of mammalian platelet activation. This analysis can be carried out on platelets isolated from a tissue or physiological fluid such as human blood, e.g., the first and second samples may comprise whole blood or platelet-rich plasma.

These results demonstrate that the present invention provides a rapid and uncomplicated assay for platelet activation via determination of surface antigens present or activated human platelets, and that P-selectin is a more sensitive and specific marker than the GP IIb-IIIa complex or than fibrinogen for platelet activation.

Thus, the present assay can be used to evaluate, monitor and stage platelet activation-related events associated with acute coronary syndromes, and in restenosis following percutaneous transfemoral coronary angioplasty (PTCA). With respect to the role of circulating activated platelets in these states, see, for example, I. Weinberger et al., Am. J. Card., 70, 981 (1992); E. Minar et al., Card., 170, 767 (1989); R.S. Schwartz et al., J. Am. Col. Card., 19, 267 (1992) and D. Tshoepe et al., Circ., 88, 37 (1993).

Brief Description of the Figures

Figure 1 is a schematic depiction of one embodiment of the present assay.

Figure 2 is a graph depicting the fluorescence intensity of P-selectin in resting platelets detected in the present assay using various diluted anti-P-selectin antibody (CD62) and IgG1 (n = 2) preparations. Platelets from two normal donors were sampled and tested in the assay using various dilutions of CD62 as indicated. Circles represent nonspecific binding (1gG1), whereas circles in bold represent specific binding (CD62). Fluorescence intensity was log-transformed.

Figure 3 is a graph depicting the fluorescence intensity in diluted resting platelets detected in the present assay using anti-P-selectin antibody (n = 2), wherein samples from two normal donors were tested using CD62 diluted at 1:20 in volume. Fluorescence intensity was log-transformed and the coefficient r was computed using linear regression.

Figure 4 is a graph depicting fluorescence intensity in resting platelets detected in the present assay using various dilutions of CD62 (n = 2). Platelets from two normal donors were sampled and tested by the present assay. Log-transformed fluorescence intensity was used in the computation of the coefficient r using linear regression.

Figure 5 is a graph depicting the fluorescence intensity in ADP-stimulated platelets in plasma detected using the present assay (n = 2). Platelets were sampled from two normal donors and tested by the assay. Both phycoerythrin (PE)-conjugated and unconjugated CD62 were diluted to the same concentration. Closed circles represent the fluorescence intensities detected using PE-conjugated CD62, while triangles represent the values detected using unconjugated CD62, and the open circles represent the levels of fluorescence intensity detected by using unconjugated CD62 and PE-conjugated CD62.

Figure 6 is a graphic correlation of the present assay with flow cytometric analysis in determination of P-selectin in platelets in platelet concentrates (n = 20). Platelets were sampled from 20 normal donors' platelet concentrates using the present assay and flow cytometric analysis simultaneously. A value of r = 0.936 was obtained from the linear regression, indicating the positive association between the two assays ($p < 0.001$).

Figure 7 is a graph depicting the platelet aggregation slopes with stimulating doses of ADP as measured in an aggregometer (n = 3). Platelet aggregation in plasma was performed in triplicate by aggregometry. The slopes were the means of the steepest slopes of aggregation tracing curves in response to each dose of ADP.

Figure 8 is a graph demonstrating association between the expression of P-selectin and stimulating concentrations of ADP assayed in the present assay (n = 3). Final concentrations of ADP in platelets in plasma were used.

Figure 9 is a depiction of the time course change of platelet reactivity to ADP as measured by aggregation in aggregometer and by expression of P-selectin as detected by the present assay (n = 3). Platelets from three normal donors were sampled and assayed by aggregometry and by the present assay simultaneously. The hour indicated is the storage time of platelets after blood was drawn. The steepest slopes of aggregation-tracing curves and log-transformed fluorescence intensity were used.

Figure 10 depicts the correlation of platelet aggregation by aggregometry with the expression of P-selectin as determined by the present assay in ADP-stimulated platelets in plasma (n = 3). Platelets from three normal donors were assayed in triplicate by aggregometry and by the present assay simultaneously. The doses of ADP used are as indicated. Coefficient r was computed using linear regression.

Figure 11 depicts the correlation of expression of P-selectin by the present assay with aggregation by aggregometry in ADP-stimulated platelets in plasma at time intervals (n = 3). A dose of 2.5 μM of ADP was used in the determination of aggregation by aggregometry and by the present assay in platelets in plasma sampled from three normal donors. Values are the means ± standard deviation (SD) of three samples in triplicate. Coefficient r was computed using linear regression.

Figure 12 depicts expression of GPIIb-IIIa complex and fibrinogen on the surface of ADP-stimulated platelets in plasma (n = 3). ADP-stimulated platelets in plasma from three normal donors were sampled and assayed by the present assay. Values are the means ± SD of three samples in triplicate.

Detailed Description of the Invention

Monoclonal antibodies and polyclonal antibody preparations comprising fluorescent labels or binding sites for ligands comprising fluorescent labels are commercially available, available to the art or preparable by art-recognized procedures. Representative murine anti-P-selectin antibodies are listed in Table I, below.

Table I

Anti-P-selectin Antibody	Label	Reference
S12	Fluorescein or phyoerythrin	R.E. Scharf et al., Arteriosclerosis and Thrombosis, 12, 1475 (1992); R.P. McEver et al., J. Biol. Chem., 259, 9799 (1984).
	---	P.E. Stenberg et al., J. Cell. Biol., 101, 880 (1985).
KC4	---	S.C. Hsu-Lin et al., J. Biol. Chem., 259, 9121 (1984); R. Bonfanti et al., Blood, 73, 1109 (1989).
AC1.2; 1-18, 2-15, 2-17	---	E. Larsen et al., Cell, 59, 305 (1989).
CD62	Phyoerythrin or no label	Becton-Dickinson
G1	---	S.A. Hamburger et al., Blood, 75, 550 (1990).

Unlabelled antibodies can be conjugated to fluorescent labels such as fluorescein isocyanate (FITC) by standard techniques. See, for example, S.J. Shattil et al., Blood, 70, 307 (1987) and J.W. Goding et al., Monoclonal Antibodies: Principles and Practice - Production and Application of Monoclonal Antibodies in Cell Biology, Biochemistry and Immunology, Academic Press, London (1986) at pages 255-280. Alternatively, the antibodies can be prepared as biotinylated conjugates and

reacted with phycoerythrin-streptavidin as taught by Goding, ibid., McEver et al., ibid., and S.J. Shattil et al., J. Biol. Chem., 260, 11107 (1985). Polyclonal anti-P-selectin antibody preparations can be prepared and detected as taught by P.E. Stenberg, J. Cell Biol., 101, 880 (1985).

Although ADP is a preferred platelet activation-aggregation agonists, other useful agents for platelet activation include thrombin, serotonin, collagen and throboxane, as well as bioactive subunit polypeptides thereof.

The erythrocytic lytic agent of step d(i) of the present invention is preferably formic acid. The lytic agent may also contain a stabilizer that serves to increase the shelf life of the agent.

In a preferred embodiment of the invention, the leukocyte stabilizer is a combination of sodium carbonate, sodium sulfate and sodium chloride. Preferably, the three components are in aqueous solution. The leukocyte stabilizer may further comprise a stabilizing agent which serves to extend the shelf life of the solution.

Preferably, the cell membrane fixative employed in step d(iii) is paraformaldehyde. Although paraformaldehyde is the preferred fixative, several other sell membrane fixatives are known to those of skill in the art. The cell membrane fixative may further comprise a buffer solution.

Preferably, step (d) of the assay is carried out using automated instrumentation which vortexes the samples and rapidly adds the recited reagents. The use of such automated instrumentation shortens the preparation time of samples and thus allows a more rapid response in urgent clinical situations. One example of such an instrument is the Q-Prep machine manufactured by Coulter® Corporation, Hialeah, FL. The Q-Prep instrument consists of a matched, three reagent system that provides a gentle, no-wash erythrocyte lysing and fixing system which maintains leukocyte morphology and cell surface integrity. The use of platelets prepared in this manner offers an alternative to the use of PRP, since the results obtained using whole blood that has been treated in the Q-prep machine correlate to those obtained using PRP. See Examples 7 and 8.

The invention will be further described by reference to the following detailed examples, wherein adenosine diphosphate (ADP, Catalog No. 885-3), paraformaldehyde (Catalog No. 62H0174) and other chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, MO). Phycoerythrin (PE)-conjugated (Catalog No. 348107) and pure (Catalog No. 348100) murine monoclonal anti-human

platelet P-selectin antibodies (CD62) and PE-conjugated isotype specific mouse IgG1 (Catalog No. 340013) were purchased from Becton-Dickinson (Mountain View, CA). FITC-conjugated murine monoclonal anti-human platelet GPIIb-IIIa antibody (CD41a) (Catalog No. 0649) was obtained from AMAC (Westbrook, ME). FITC-conjugated sheep anti-human fibrinogen antibody (Catalog No. K90056F) was obtained from BIO-DESIGNE (Kennebunkport, ME). Antibodies were diluted using 1% fetal calf serum phosphate-buffered saline (PBS) solution.

Ten aspirin-free normal donors (age: 24-41, male: 5, female: 5) were recruited through the healthy donor center at Mayo Clinic. Blood was drawn in a 21 gauge butterfly needle and a plastic syringe and collected using 15 ml polypropylene centrifuge tubes (Corning Inc.) containing 1:6 (for platelet activation) and 1:9 (for platelet aggregation) volumes of acid citrate dextrose (ACD). Blood samples were centrifuged at 250 xg for 10 minutes at 15°C in a Mistral 3000-i centrifuge with rate of 5 for brake and acceleration settings, respectively, to obtain platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was prepared by further centrifugation of the remaining blood at 1500 xg for 10 minutes. Platelet counts were performed on the Coulter Counter (Coulter Electronics, Inc.) and PRP was adjusted with PPP to a constant count of 3.0 x 10 e-8/ml.

Platelet aggregation studies were performed at 37°C on a dual channel aggregometer (Dayton Dual Channel Aggregation Module), at a stirring speed of 900 rpm. Optical density for PRP and PPP was set at 10% and 90%, respectively. Adenosine diphosphate (ADP) (0.05 ml) was added to 0.45 ml of stirred suspension of PRP up to the final concentration as shown. The maximal or steepest slope of the aggregation tracing curve was measured.

The maximal slope of the platelet aggregation tracing curve was computed using the equation: $Dfi = f(ti)-f(ti-1)/ti-t(i-1) = h\ max(cm)/t(min)$, where h max is the height of the steepest slope of the curve in centimeters, and t is the time of the steepest slope of the curve in minutes. Coefficient (r) values were computed using linear regression. In some computations, logarithm-transformed data were used.

The fluorescence measurements were obtained using the IDEXX Fluorescence Concentration Analyzer (FCA) machine (IDEXX Laboratories, Inc., Westbrook, ME). This instrument uses a specially designed (96-2311) 2 mm diameter filter membrane-bottomed plate (Fluoricon assay plate) that separates antibody-bound cells from non-bound antibody in solution by applying a vacuum (0-

25 mmHg) from below the membrane. The total cell/antibody-bound fluorescence is determined by front-surface fluorimetry. The instrument has a fluorimeter capable of exciting and reading at several wavelengths (400/450 nm-590/620 nm).

Example 1.
Fluorescence-conjugated Immunobinding Assay for Platelet Activation

Ten µl of phosphate buffer saline (PBS, 0.01 M, pH 7.4, as baseline) or ADP in a series of concentrations (10 µM, 25 µM, 50 µM, 100 µM and 150 µM) were added to a 90 µl sample of PRP in round-bottomed polystyrene tubes and allowed to incubate at room temperature for five minutes. Eighty µl of mixed sample was immediately added to a 1 ml final concentration of 1% of paraformaldehyde PBS solution in 1.5 ml vials, and the samples were incubated at 4°C for four hours. The fixed samples were washed twice by centrifugation in a microcentrifuge using PBS solution. The washed platelets were diluted to 8.5 x 10 e-7/ml in PBS solution.

A 20 µl aliquot of diluted samples was placed in 96 well plates. A 20 µl aliquot of 1:20 diluted CD62, or 1:40 diluted CD41a, or 1:50 diluted antifibrinogen Ab was added and the samples were incubated in the dark at room temperature for 30 minutes. The cells in the Fluoricon assay plates were concentrated and washed three times in washing buffer (1% Tween PBS) by applying a vacuum membrane from below the plates of 25 mm Hg in the Pandex FCA machine. This step removed any unbound antibody and free fluorescence marker from the complexes. Antibody-bound fluorescence was determined by reading plates at a gain of 1 for CD41a and antifibrinogen Ab and of 10 for CD62 on the FCA instrument at the appropriate wavelength. Data was recorded as relative fluorescence units (FU), after subtracting the blank.

In experiments designed to determine the optimal dilution of PRP and of fluorescence-conjugated antibodies, the same total volume, with varying dilutions of PRP and the antibodies, was used. For the determination of the time course change of platelet reactivity to ADP as measured by aggregation and by expression of P-selectin, PRP was stored at room temperature in the centrifuge tubes after being diluted to a constant concentration with PPP. At each time point, PRP was withdrawn from the stock tubes and added to test tubes and the activation and aggregation assays performed as described above. For the comparison of the present

assay to the flow cytometric analysis, samples were prepared as described in flow cytometric analysis example, and 100 µl of the samples were placed in Fluoricon plates and read on the FCA instrument.

To select the optimal conditions for the assay, various dilutions of antibody and antigen (platelets) were tested in the present assay. The selection of optimal conditions was based on selecting the concentration of the antibody that was on the steepest slope of the dilution curve and from which the most specific and the least non-specific reactions were obtained. Optimal conditions were obtained using anti-P-selectin antibody at a 20-fold dilution in volume or at an antibody/platelets ratio of 0.04 µg/1.5 x 10 e-6 cells. At this dilution, the specific reaction was greatest while demonstrating the lowest non-specific reaction (Figure 2) and a wide range of concentrations of P-selectin was detectable in a dose-dependent manner (Figure 3). Furthermore, it was in the middle of a linear dilution curve (Figure 4), which makes the assay specific and sensitive. When the same amount of unconjugated monoclonal antibody to P-selectin was added to platelets in plasma followed by the addition of fluorescence-conjugated P-selectin antibody, fluorescence intensity of the platelets was decreased by 50%, indicating a satisfactory competitive inhibition of binding (Figure 5).

Expression of P-selectin in four fixed platelet aliquots (mean levels: 56.00 to 60.00 FU/1.5 x 10 e-g cells) from the same healthy subject were determined in ten replicates (for intra-assay variability) and in triplicates (for inter-assay variability) in multiple separate assays. The intra-assay CVs for the means of ten replicates ranged from 3.45% to 10.78% (mean: 6.97%). The time-based interassay CVs for the means of triplicates ranged from 5.93% to 12.39% (mean: 8.11%). The sample-based inter-assay CVs for the means of triplicates ranged from 2.82% to 13.99% (mean: 6.17%).

After incubation with CD62 for 30 minutes, a drop of platelets in plasma was transferred to a slide. Platelets were then evaluated by microscopy. ADP-activated platelets became larger, developed protrusions, and changed to a spherical shape under light microscopy. These activated platelets demonstrated red fluorescence under fluorescence microscopy.

Example 2.

Flow Cytometric Analysis

Platelet concentrates were obtained from the Mayo Clinic blood bank within 24 hours of collection of blood from volunteer donors. Platelet concentrate (PC) was prepared by collecting blood (450 ± 45 ml) from random donors in 63 ml of CPD in a pyrogen-free (Fenwal Laboratories, Morton Grove, IL) quad blood collection pack with an attached satellite bag containing 100 ml of ADSOL solution. After blood collection, whole blood was centrifuged for 5.2 minutes at 1400 g's at 20-24°C. The platelet-rich plasma was pressed into an empty satellite bag, leaving approximately 50 ml of PC. The PC was left undisturbed for 1 hour, was resuspended on a platelet rotator, and stored on a horizontal flatbed shaker. Twenty individual PC units were sampled after 24 hours of storage.

Samples were prepared for analysis by fixing 100 µl of platelets with 1 ml cold 1% paraformaldehyde for 1 hour at 4°C. The platelets were washed (x2) with phosphate-buffered saline/EDTA (PBS/EDTA), the pellet was resuspended in 1 ml PBS/EDTA and stored at 4°C in the dark. The following day (within 24 hours) 50 µl of the resuspended platelets were labeled with 10 µl of monoclonal antibody CD41 (AMAC, Inc., Westbrook, ME). After a 10-minute incubation in the dark at room temperature (RT), 20 µl of MoA6 (Becton-Dickinson, San Jose, CA) was added and incubated 20 more minutes in the dark at 25°C. The sample was then washed (x1) with PBS/EDTA and the pellet was resuspended in 1 ml of cold 1% paraformaldehyde and stored at 4°C in the dark for flow cytometric analysis. All samples were analyzed within 6 hours of labeling. Samples were analyzed on a flow cytometer (FACScan, Becton-Dickinson, Mountain View, CA) within 6 hours of labeling. The percentage of platelets expressing P-selectin (the percentage of activated platelets) was determined as described by R. Funbeer et al., Transfusion, 30, 20 (1990). The mean GPIIb-IIIa surface density was determined for the subsets of P-selectin-negative platelets and P-selectin-positive platelets. See, H.M. Rinder et al., Transfusion, 31, 409 (1991); Anesthesiology, 75, 963 (1991).

Quantitative expression of P-selectin as fluorescence intensity or as the ratio of P-selectin positively stained platelets in one-day stored platelets in platelet concentrates from 20 normal donors were determined simultaneously by the present assay and by flow cytometric analysis, respectively. Linear regression analysis of the data showed that the log-transformed fluorescence intensity of P-selectin as

determined in FCIBA was correlated with the ratio of P-selectin positively stained platelets expressed as a log-transformed ratio as measured in flow cytometric analysis ($r = 0.936$, $p < 0.001$) (Figure 6).

Example 3.
Induction of Expression of P-selectin and Aggregation in Platelets by ADP

Platelets in plasma from three healthy subjects aggregated in the aggregometer in response to ADP in a dose-dependent manner (Figure 7), and the steepest slopes of the aggregation tracing curves were correlated with increasing stimulating doses of ADP (Figure 7). P-selectin in platelets in plasma from the same three healthy subjects was also translocated to the plasma membrane of platelets in response to ADP in a dose-dependent manner, as measured by phycoerythrin-fluorescence with increasing stimulating doses of ADP, in accord with Example 2 (Figure 8).

Example 4.
Time Course Change of Platelet Reactivity to ADP

Reactivity of platelets in plasma to ADP as measured by aggregation or by expression of P-selectin from three healthy donors were measured both by an aggregometer and by the procedure of Example 1, at time intervals ranging from 1.5 to 10 hours after blood was drawn. Platelets in plasma aggregated in response to increasing stimulating doses of ADP in parallel with the expression of P-selectin at time intervals (Figure 9). The reactivity of platelets to ADP was increased at time intervals, and reached similar peak levels at the seventh hour (Figure 9).

Example 5.
Relation of ADP-induced Aggregation and Expression of P-selectin in Platelets

Data from Examples 3-4 were analyzed using linear regression. This showed that the expression of P-selectin in platelets in plasma as measured by the present assay was correlated positively with platelet aggregation of platelets in plasma as determined by aggregometry in response to ADP both on the basis of stimulating doses of ADP (Figure 10) and on the basis of time intervals (Figure 11).

Example 6.

Relation of Expression of P-selectin and Exposure of GP IIb-IIIa and Fibrinogen in Platelets in Response to ADP

To determine the alterations in the amount of GPIIb-IIIa complex and P-selectin expressed on the platelet surface and in levels of fibrinogen bound to platelets or exposed on platelets in response to the stimulation of ADP, platelets in plasma from two healthy subjects were sampled and tested in the assay of Example 1 simultaneously by using monoclonal antibodies to GPIIb-IIIa as well as with monoclonal antibodies to P-selectin, and a polyclonal antibody to fibrinogen. Detectable GPIIb-IIIa complex in platelets showed little change in response to stimulation with ADP (Figure 12). In contrast, the levels of fibrinogen bound to platelets or exposed on the surface of platelets in response to increasing doses of ADP was increased and reached a peak level at a dose od 5.0 μM of ADP (Figure 12). In platelets in plasma, the changes in the amount of detectable GIIb-IIIa complex correlated weakly with alteration in the levels of bound or exposed fibrinogen in response to the stimulating doses of ADP ranging from 0.0 to 10.0 μM ($y = 355.64 + 3.29 X$, $r = 0.45$, $p > 0.05$), and also correlated only weakly with the alteration in levels of expressed P-selectin ($y = 269.47 + 170.44 * \log (X)$, $r = 0.663$, $p > 0.05$). The alteration to levels of fibrinogen bound to platelets or exposed on the surface of platelets was correlated more strongly with changes in levels of P-selectin expressed in response to the increasing stimulating doses of ADP ranging from 0.0 to 10.0 μM ($y = 167.3 + 0.44 X$, $r = 0.858$, $P < 0.05$), suggesting the association of P-selectin with the binding or exposure of fibrinogen on ADP-stimulated platelets.

Detection of P-selectin translocation to the platelet surface as determined by the present method is a sensitive and specific measure of platelet activation, which correlates well with more complex traditional measures of this phenomenon. Although simpler cells could be employed for fluorometry, the methodology using microtiter plates with filters and the measurement of front-surface fluorimetry to measure this translocation permits measurements to be made in 96 wells as a single semiautomated procedure. For further expansion of the analytic capacity, a fluorescence analyzer with the ability to read 10 plates (960 wells) as an even more automated procedure (screen machines, IDEXX, Portland, ME) is also available. These features make the present method very practical for the study of the dynamics of platelet activation in the clinical disease states discussed above.

Example 7.

Comparison of Results Using Whole Blood and PRP and Using CD62 as a Marker of Platelet Activation.

A 6 ml sample of whole blood was isolated, divided into twenty 100 µl samples, and added to round-bottomed polystyrene tubes. Thirty µl of phosphate buffered saline (PBS, 0.01 M, pH 7.4, as baseline) or ADP (2.5 µM) were added to each sample and all were allowed to incubate at room temperature for five minutes. One hundred µl of 1% of paraformaldehyde PBS solution was added to each tube, and the samples were incubated at room temperature for fifteen minutes. Following this incubation, each sample was placed in a Q-Prep machine (Coulter® Corporation, Hialeah, FL) for a 30-second cycle. During this cycle, the sample was vortexed while a series of three reagents was added. The first reagent added was a solution of formic acid at a concentration of 1.2 mL/L. Next, a solution of sodium carbonate (6.0 g/L), sodium chloride (14.5 g/L) and sodium sulfate (31.3 g/L) was added. Finally, a solution of paraformaldehyde was added (10.0 g/L). The fixed samples were then washed twice by centrifugation in a microcentrifuge using PBS solution. The washed platelets were diluted to 8.5 x 10 e-7/ml in PBS solution. All samples were analyzed within 72 hours of fixing.

A 20 µl aliquot of each of the diluted samples was placed in 96 well plates. A 20 µl aliquot of 1:20 diluted CD62 Ab was added and the samples were incubated in the dark at room temperature for 30 minutes. The cells in the Fluoricon assay plates were concentrated and washed three times in washing buffer (1% Tween PBS) by applying a vacuum membrane from below the plates of 25 mm Hg in the Pandex FCA machine. This step removed any unbound antibody and free fluorescence marker from the complexes. Antibody-bound fluorescence was determined by reading plates at a gain of 10 for CD62 on the FCA instrument at the appropriate wavelength. Data was recorded as relative fluorescence units (FU), after subtracting the blank and is shown in Table II along with the results obtained from 20 samples of PRP that were analyzed according to the method of Example 2.

Table II.

Comparison of Results Using Whole Blood and PRP and Using CD62 as a Marker of Platelet Activation.[1]

Sample	Whole Blood		PRP	
	ADP-activated	PBS Control	ADP Activated	PBS Control
1	3326	0	2000	0
2	1704	0	1262	0
3	3228	0	5206	0
4	4158	0	3874	0
5	7870	0	6644	0
6	3792	0	3284	0
7	4594	0	3122	0
8	6588	0	4244	0
9	7024	0	9052	0
10	3762	0	6750	0

1 Data is recorded as absolute fluorescence units (FU).

As can be seen from Table II, the degree of platelet activation achieved utilizing whole blood correlates well with the degree of activation achieved when utilizing platelet-rich plasma. Furthermore, there was no activation observed in the control samples of either whole blood or PRP.

Example 8

Comparison of Results of Using Whole Blood and PRP and Using FITC Labeled Fibrinogen as a Marker for Platelet Activation

A 6 ml sample of whole blood was isolated, divided into twelve 100 µl samples, and added to round-bottomed polystyrene tubes. Thirty µl of fibrinogen, labeled with FITC (1:100 dilution) were added to each sample. Subsequently, 30 µl of either ADP (2.5 µM) or phosphate buffer saline (PBS, 0.01 M, pH 7.4, as baseline)was added to each tube and the samples were allowed to incubate at room temperature for five minutes. One hundred µl of 1% of paraformaldehyde PBS solution was added to each tube, and the samples were incubated at room temperature for fifteen minutes. Following this incubation, each sample was placed in a Q-Prep machine (Coulter® Corporation, Hialeah, FL) for a 30-second cycle. During this

cycle, the sample was vortexed while a series of three reagents was added. The first reagent added was a solution of formic acid at a concentration of 1.2 mL/L. Next, a solution of sodium carbonate (6.0 g/L), sodium chloride (14.5 g/L) and sodium sulfate (31.3 g/L) was added. Finally, a solution of paraformaldehyde was added (10.0 g/L). The fixed samples were then washed twice by centrifugation in a microcentrifuge using PBS solution. The washed platelets were diluted to 8.5 x 10 e-7/ml in PBS solution. All samples were analyzed within 72 hours of fixing.

A 20 µl aliquot of diluted samples was placed in 96 well plates. A 20 µl aliquot of 1:20 diluted CD62 Ab was added and the samples were incubated in the dark at room temperature for 30 minutes. The cells in the Fluoricon assay plates were concentrated and washed three times in washing buffer (1% Tween PBS) by applying a vacuum membrane from below the plates of 25 mm Hg in the Pandex FCA machine. This step removed any unbound antibody and free fluorescence marker from the complexes. Antibody-bound fluorescence was determined by reading plates at a gain of 10 for CD62 on the FCA instrument at the appropriate wavelength. Data was recorded as relative fluorescence units (FU), after subtracting the blank and is shown in Table III along with the results obtained from Example 6 wherein PRP was used. Table IV shows the data that was obtained by following the above protocol employing FITC labeled fibrinogen as a 1:50 dilution.

Table III.

Comparison of Results Using Whole Blood and PRP and Using FITC Labeled Fibrinogen at a 1:100 Dilution as a Marker of Platelet Activation.[2]

Sample	ADP	PBS
Sample 1 (PRP)	4226	0
Sample 2 (PRP)	3972	0
Sample 3 (PRP)	4208	12
Sample 4 (Whole Blood)	3624	0
Sample 5 (Whole Blood)	3454	0
Sample 6 (Whole Blood)	3640	0

2 Data recorded as absolute fluorescence units (FU).

As demonstrated by the data on Table III, fibrinogen binding is quite obvious and readily detectable. Although the numbers for platelet-rich plasma (PRP) are somewhat higher than that obtained using whole blood, the use of whole blood provided an equally suitable indication of platelet activation. As can be seen by comparing the data contained in Tables III and IV (below), the utilization of a higher concentration of FITC labeled fibrinogen is not necessary to elicit a detectable response from either the whole blood samples or the platelet rich plasma samples.

Table IV.

Comparison of Results of Whole Blood and PRP Methods Using FITC Labeled Fibrinogen at a 1:50 Dilution as a Marker of Platelet Activation.[3]

Sample	ADP	PBS
Sample 1 (PRP)	11838	150
Sample 2 (PRP)	12184	80
Sample 3 (PRP)	11862	98
Sample 4 (Whole blood)	3772	172
Sample 5 (Whole blood)	7238	216
Sample 6 (Whole blood)	6238	190

[3] Data recorded as absolute fluorescence units (FU).

All publications, patents and patent documents are incorporated by reference herein, as though individually incorporated by reference. The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A fluorometric method for measuring endogenous platelet activation in a sample of platelets comprising:
   (a) obtaining a sample of physiological fluid from a patient whose platelet activation is to be determined and dividing it into a first sample and a second sample;
   (b) adding an amount of an activation agonist to said first sample for a period of time effective to maximally activate the activatable platelets in said first sample; while maintaining the second sample for an equivalent period of time;
   (c) adding an amount of an agent to said first sample effective to stop the platelet activation reaction and to partially fix the activated platelets in said first sample, while maintaining the second sample for an equivalent period of time;
   (d) Vortexing the samples, while sequentially adding to each sample:
      (i) an amount of an erythrocytic lytic agent effective to lyse the red blood cells in the samples;
      (ii) an amount of a leukocyte stabilizer effective to stop the action of the lytic agent; and
      (iii) an amount of a cell membrane fixative effective to completely fix the platelets and to stabilize the samples;
   (e) forming binary labelled complexes with the activated platelets in each sample by adding to each sample an amount of
      (i) an anti-P-selectin antibody conjugated to a fluorescent label; or
      (ii) an anti-P-selectin antibody conjugated to a binding site for a detectable fluorescent label followed by a detectable fluorescent label which specifically binds to said binding site; and
   (f) determining the fluorescence of the binary labelled complexes in each sample, wherein a ratio of the fluorescence of said second sample to said first sample provides a measure of the extent of a platelet activation in said second sample.

2. The method of claim 1 wherein the mammal is a human.

3. The method of claim 2 wherein said first and second samples comprise whole blood.

4. The method of claim 3 wherein the activation agonist is adenosine 5'-diphosphate.

5. The method of claim 1 wherein the anti-P-selectin antibody is monoclonal antibody CD62.

6. The method of claim 5 wherein said monoclonal antibody CD62 is fluoresceinated.

7. The method of claim 1 wherein the agent in step (c) is paraformaldehyde.

8. The method of claim 1 wherein the erythrocytic lytic agent is formic acid.

9. The method of claim 1 wherein the leukocyte stabilizer is an aqueous solution comprising sodium carbonate, sodium sulfate and sodium sulfate.

10. The method of claim 1 wherein the cell membrane fixative is formaldehyde.

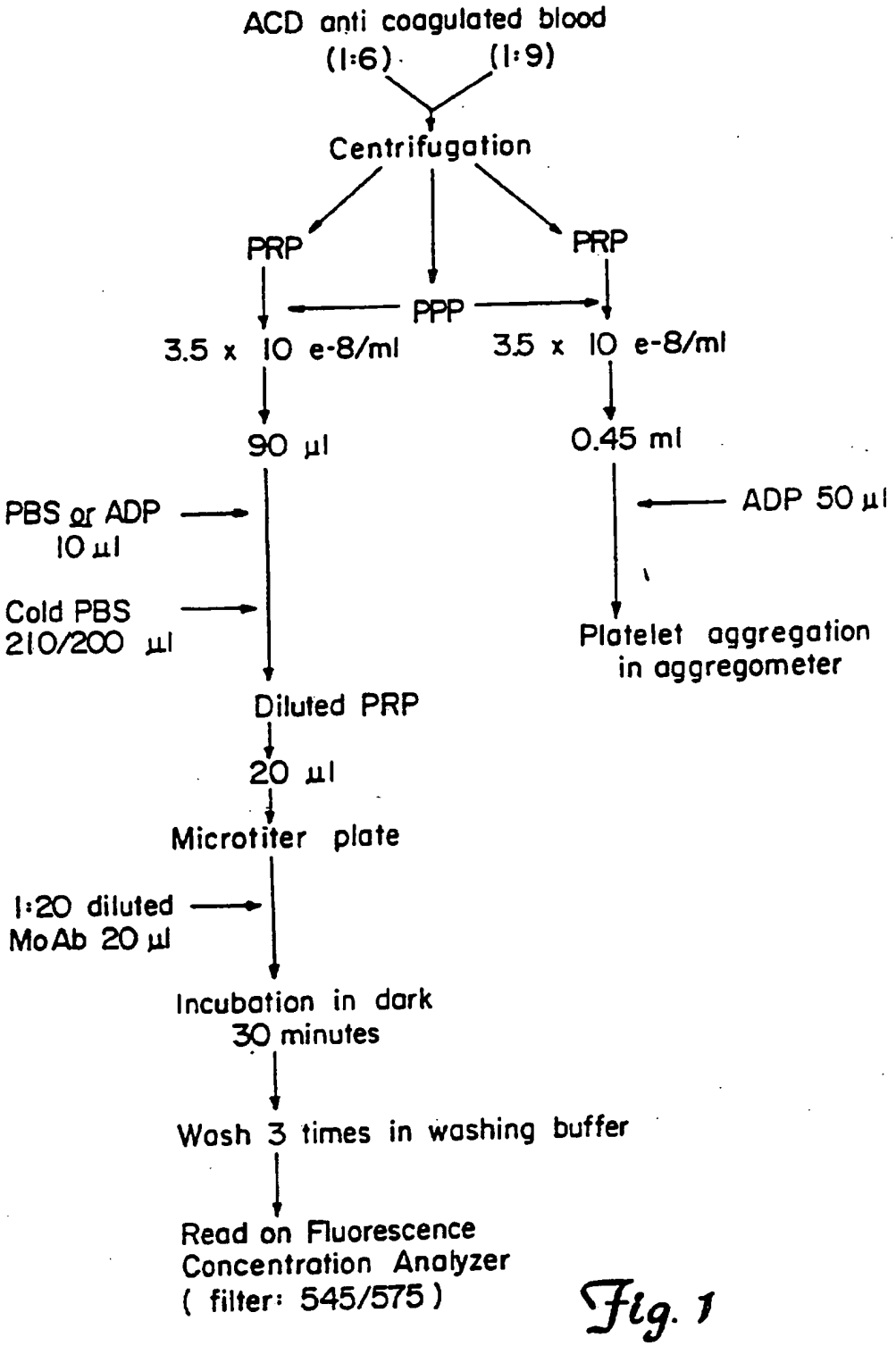
ACD anti coagulated blood
(1:6)
(1:9)
Centrifugation
PRP
PRP
PPP
3.5 x 10 e-8/ml
3.5 x 10 e-8/ml
90 µl
0.45 ml
PBS or ADP
10 µl
ADP 50 µl
Cold PBS
210/200 µl
Platelet aggregation
in aggregometer
Diluted PRP
20 µl
Microtiter plate
1:20 diluted
MoAb 20 µl
Incubation in dark
30 minutes
Wash 3 times in washing buffer
Read on Fluorescence
Concentration Analyzer
(filter: 545/575)

Fig. 1

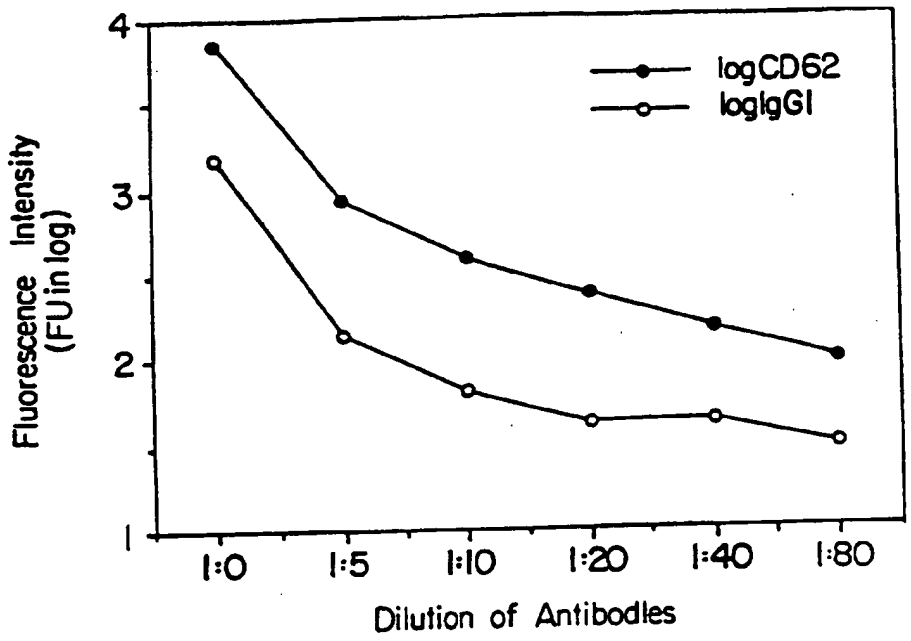
4
3
2
1
Fluorescence Intensity
(FU in log)
logCD62
logIgGI
1:0
1:5
1:10
1:20
1:40
1:80
Dilution of Antibodies

Fig. 2

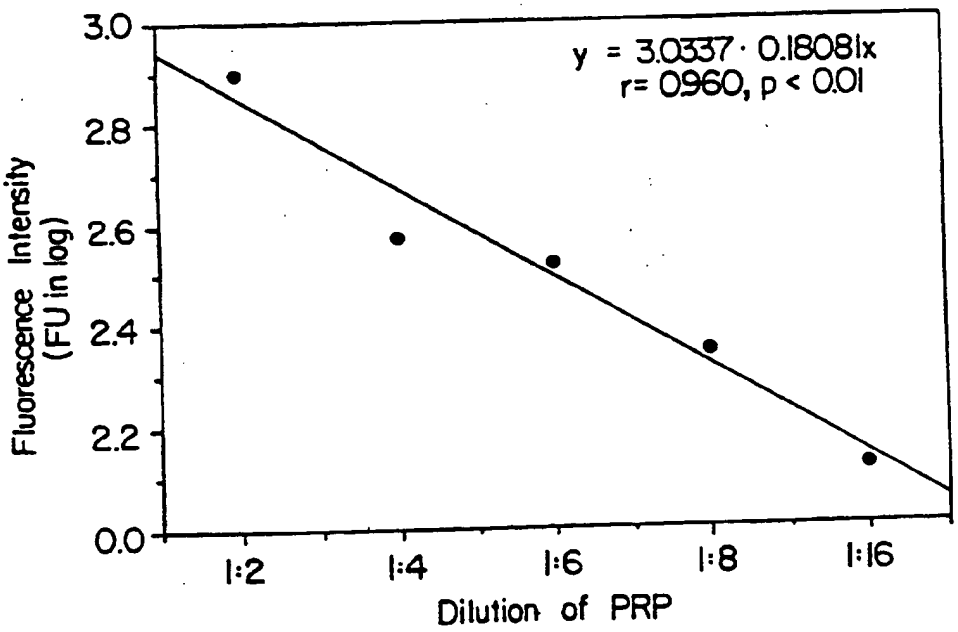
3.0
2.8
2.6
2.4
2.2
0.0
Fluorescence Intensity
(FU in log)
y = 3.0337 · 0.1808lx
r= 0.960, p < 0.01
1:2
1:4
1:6
1:8
1:16
Dilution of PRP

Fig. 3

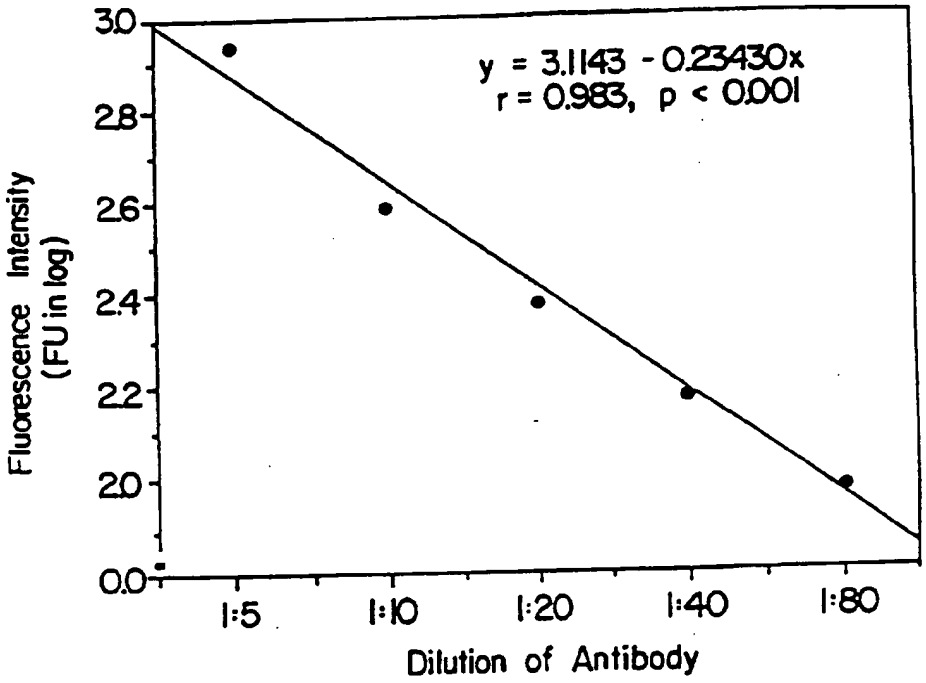
y = 3.1143 - 0.23430x
r = 0.983, p < 0.001
Fluorescence Intensity (FU in log)
3.0
2.8
2.6
2.4
2.2
2.0
0.0
1:5
1:10
1:20
1:40
1:80
Dilution of Antibody

Fig. 4

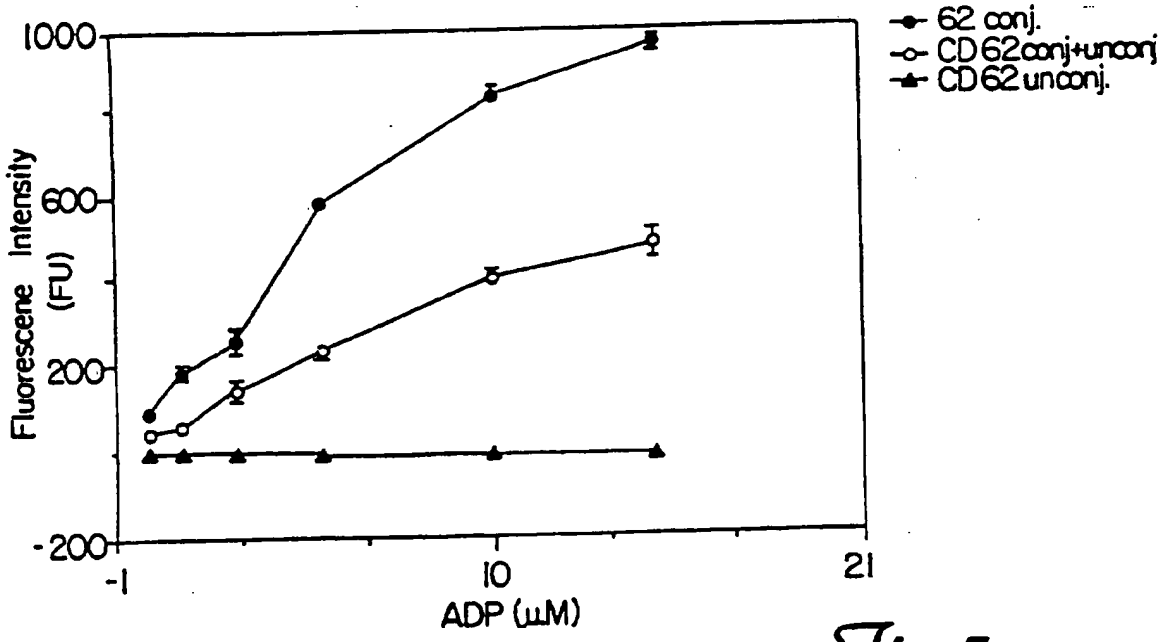
62 conj.
CD62conj+unconj
CD62unconj.
Fluorescene Intensity (FU)
1000
600
200
-200
-1
10
21
ADP (μM)

Fig. 5

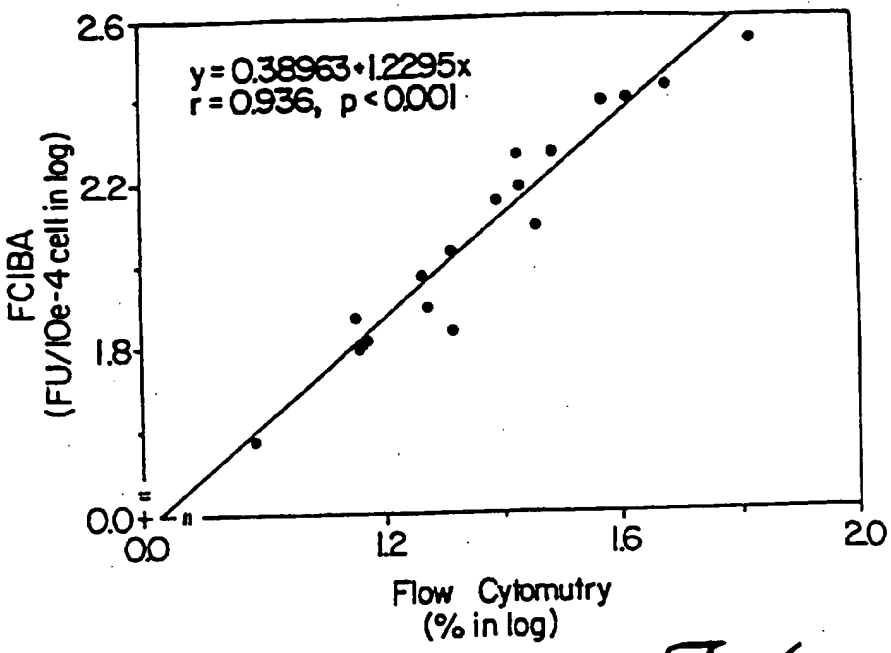
y = 0.38963 + 1.2295x
r = 0.936, p < 0.001
FCIBA
(FU/10e-4 cell in log)
2.6
2.2
1.8
0.0
0.0
1.2
1.6
2.0
Flow Cytomutry
(% in log)

Fig. 6

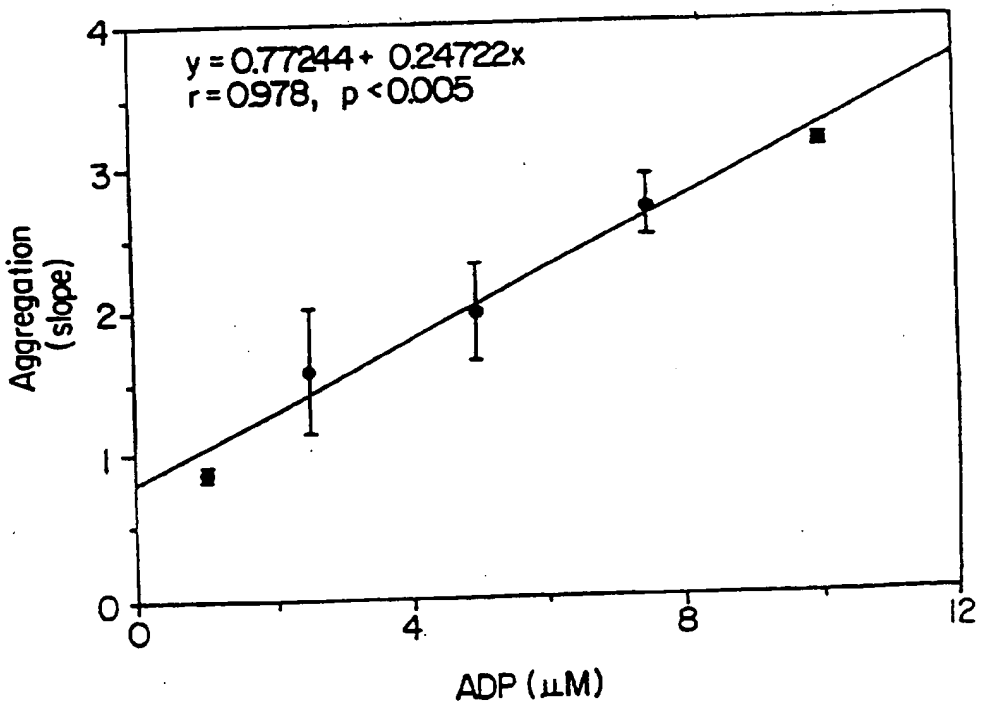
y = 0.77244 + 0.24722x
r = 0.978, p < 0.005
Aggregation
(slope)
4
3
2
1
0
0
4
8
12
ADP (μM)

Fig. 7

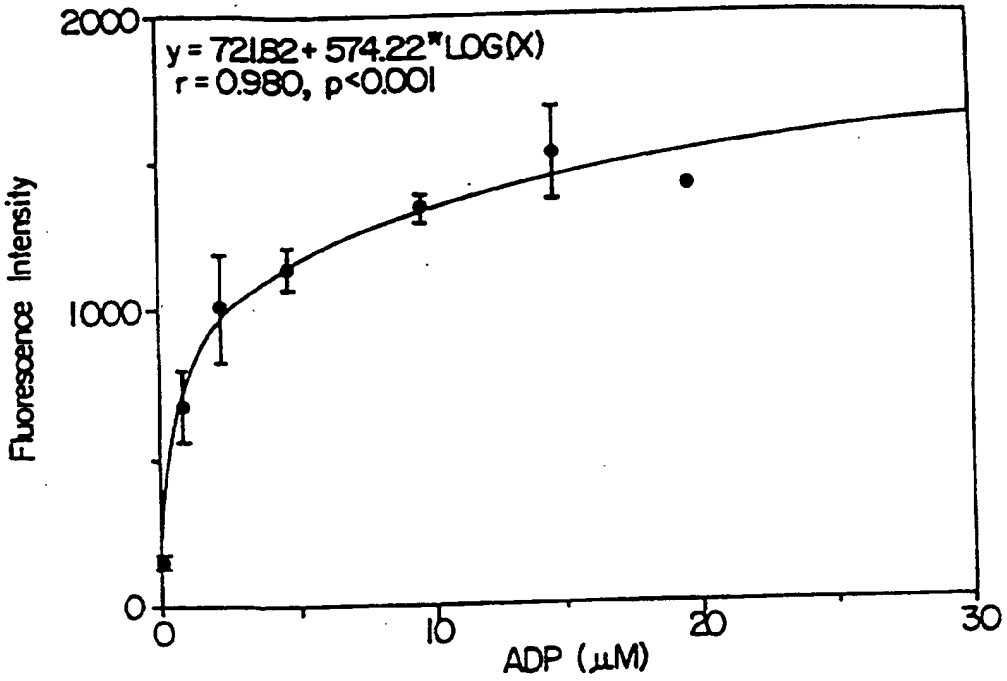
y = 721.82 + 574.22*LOG(X)
r = 0.980, p<0.001
Fluorescence Intensity
2000
1000
0
0
10
20
30
ADP (μM)

Fig. 8

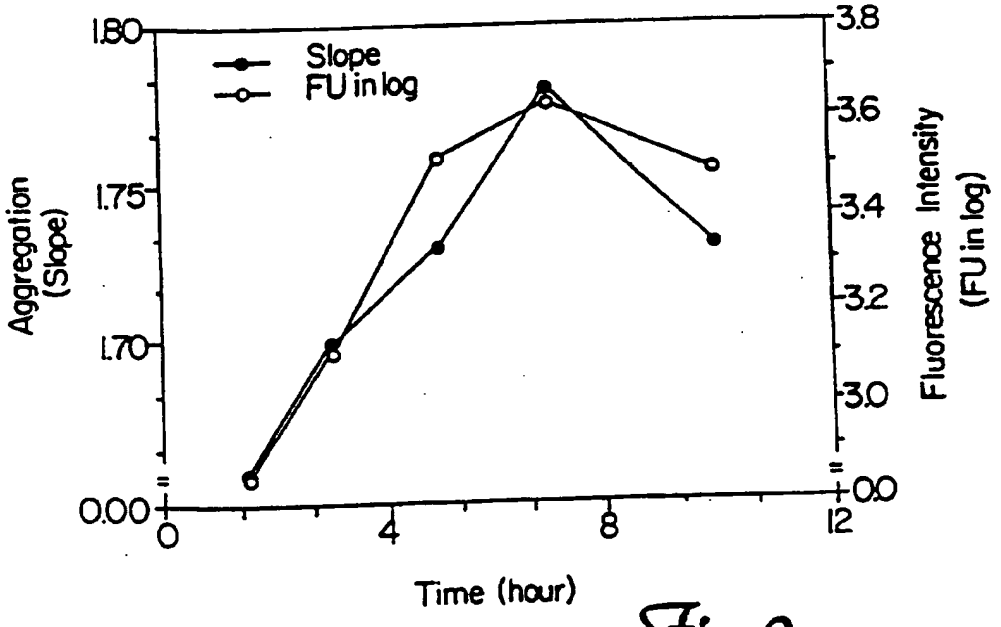
Slope
FU in log
Aggregation (Slope)
Fluorescence Intensity (FU in log)
1.80
1.75
1.70
0.00
3.8
3.6
3.4
3.2
3.0
0.0
0
4
8
12
Time (hour)

Fig. 9

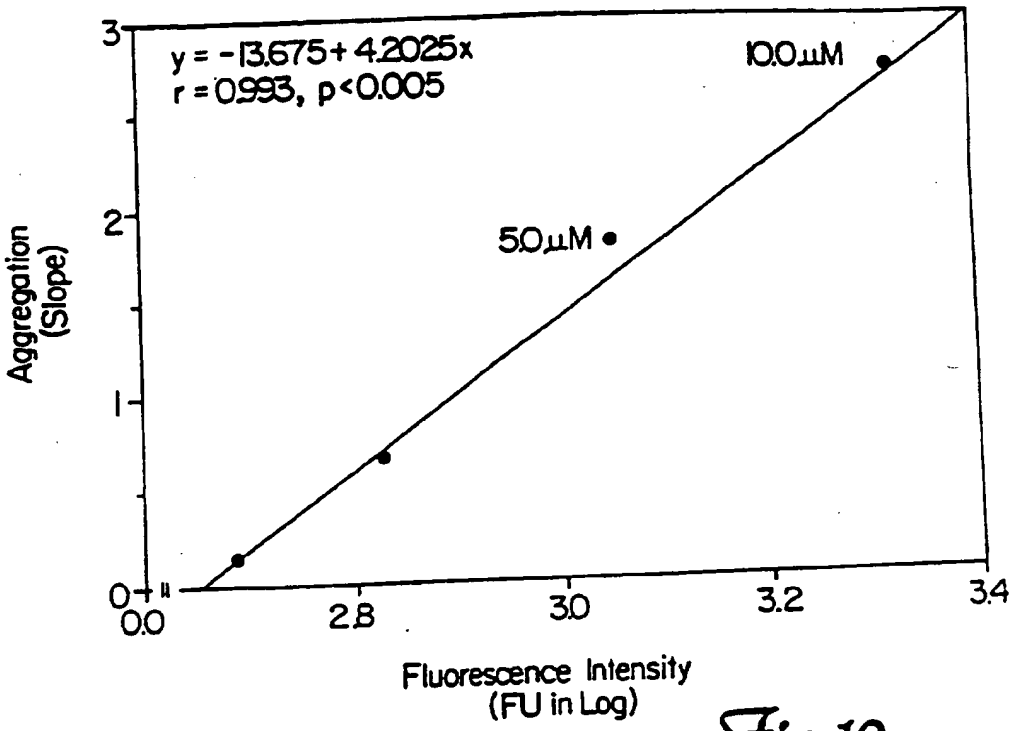
y = -13.675 + 4.2025x
r = 0.993, p<0.005
10.0 µM
5.0 µM
Aggregation (Slope)
Fluorescence Intensity (FU in Log)
0.0
2.8
3.0
3.2
3.4
0
1
2
3

Fig. 10

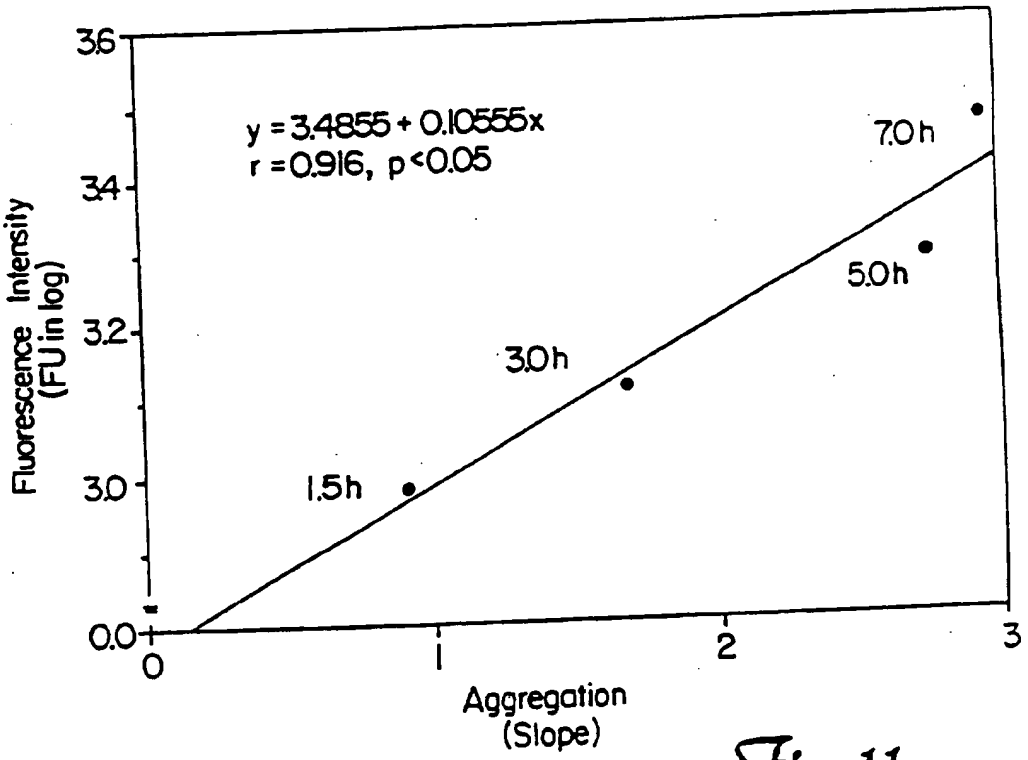
y = 3.4855 + 0.10555x
r = 0.916, p<0.05
7.0 h
5.0 h
3.0 h
1.5 h
Fluorescence Intensity (FU in log)
Aggregation (Slope)
0.0
3.0
3.2
3.4
3.6
0
1
2
3

Fig. 11

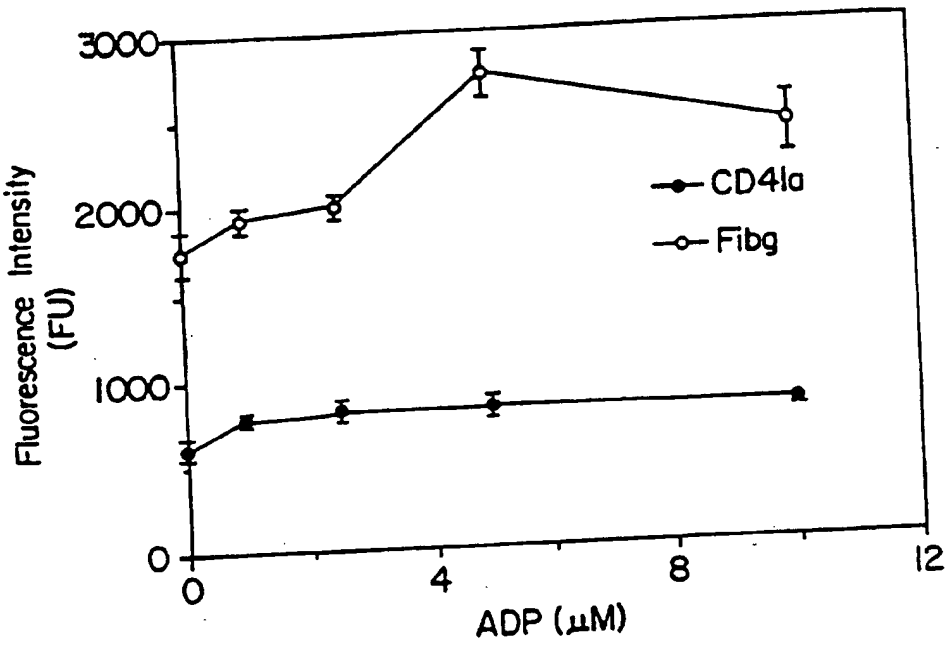
3000
2000
1000
0
Fluorescence Intensity (FU)
CD41a
Fibg
0
4
8
12
ADP (μM)

Fig. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14041

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/53, 33/533
US CL :435/7.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.21 , 7.24, 962; 436/501, 63, 69

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
search terms: platelet, activate, anti-P-selectin, cd62, fluorescence

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,783,330 (FURIE ET AL.) 08 November 1988, column 1, lines 28-56, column 8, lines 15-22.	1-10
A	US, A, 5,030,554 (QUINTANA ET AL.) 09 July 1991, column 4, lines 29-62.	1-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 22 FEBRUARY 1996	Date of mailing of the international search report 01 MAR 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Deborah Frieze LORA M. GREEN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*